

# An internal RNA element in the P3 cistron of *Wheat streak mosaic virus* revealed by synonymous mutations that affect both movement and replication

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Multiple synonymous substitution mutations in the *Wheat streak mosaic virus* P3 cistron did not affect translation *in vitro* but rendered the virus incapable of systemic infection. Multiple synonymous substitutions in the cylindrical inclusion cistron did not alter infectivity or *in vitro* translation. To assess replication and movement phenotypes, P3 mutations were placed in context with a GUS reporter gene. GUS activity measured in barley protoplasts 36 h post-transfection indicated that mutants with synonymous substitutions in P3 retained the ability to replicate at 22–80 % of wild-type levels. Almost no GUS activity was detected in protoplasts transfected with a P3 frame-shift mutant. Histochemical GUS assays conducted 3 days post-inoculation (p.i.) revealed genomes with multiple synonymous substitutions in P3, which were able to establish infection foci limited to small clusters of cells that increased in size only slightly by 5 days p.i. Infection foci produced by wild-type *Wheat streak mosaic virus*-expressing GUS were much larger at 3 days p.i. and had coalesced by 5 days p.i. No GUS activity was detected in plants inoculated with the frame-shift mutant bearing GUS. Three of four mutants, each with a single synonymous substitution in the 3'-proximal half of the P3 cistron, were wild-type with respect to systemic infectivity. A model RNA secondary structure obtained for the region was disrupted by the debilitating single mutation but not by the other three single mutations. Collectively, these results identify an internal RNA sequence element in the P3 cistron that affects both replication and movement of the viral genome.

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## INTRODUCTION

Species of the family *Potyviridae* have single-stranded, positive-sense RNA genomes of ~9–11 kb (Shukla *et al.*, 1998), encoding a single large polyprotein that is cleaved into 8–10 mature proteins by three viral-encoded proteinases (P1, HC-Pro and NIa). Mutational analyses have indicated that most, if not all, potyviral proteins are multifunctional. Furthermore, it appears that basic viral functions, including replication and movement, are affected by more than one viral-encoded protein (for review see Urcuqui-Inchima *et al.*, 2001).

Compared with other potyviral gene products the function of P3 is less well understood. The P3 cistron encodes a protein that is excised from the polyprotein by proteinase activities of HC-Pro at the amino-proximal end (Oh & Carrington, 1989; Carrington & Herndon, 1992) and NIa at the carboxy-proximal end (Carrington & Dougherty, 1987; Dougherty *et al.*, 1989). Insertional mutagenesis of the *Tobacco vein mottling virus* (TVMV) P3 cistron abolished

infectivity and detectable replication in protoplasts (Klein *et al.*, 1994). Immunogold-labelled antibodies raised against the TVMV P3 protein localized to the cylindrical inclusions (CIs) of infected cells, suggesting that the P3 protein interacts with the potyviral CI protein during early stages of inclusion formation (Rodríguez-Cerezo *et al.*, 1993). *In vitro* binding assays (Merits *et al.*, 1999) determined that the *Potato virus A* (PVA) P3 protein interacted with viral-encoded proteins of the putative replication complex (CI, NIa-VPg, NIa-Pro and NIb), although in yeast two-hybrid assays the same researchers reported that PVA P3 protein interacted only with NIb (Merits *et al.*, 1999). Similar *in vitro* and *in vivo* interaction assays revealed that P3 of *Wheat streak mosaic virus* (WSMV) is capable of binding to itself, P1, HC-Pro and CI (Choi *et al.*, 2000a).

The potyviral P3 cistron also affects pathogenesis. Symptom severity phenotype of *Plum pox virus* (PPV) on *Nicotiana clevelandii* mapped to the P3-6K1 region but was independent of virus accumulation (Sáenz *et al.*, 2000). The ability of *Pea seed-borne mosaic virus* (PSBMV) to infect a resistant

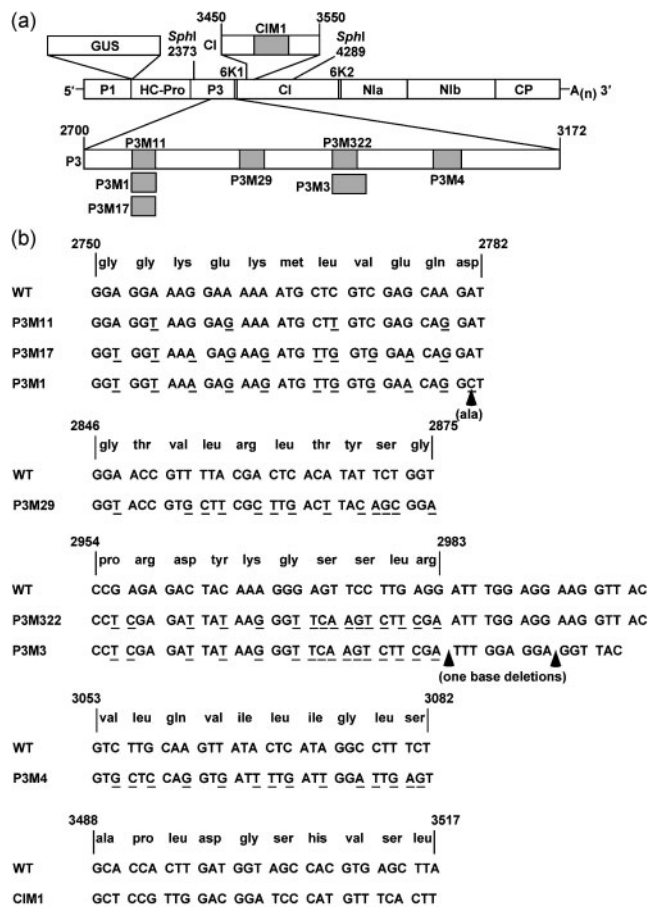
pea line was dependent upon the P3-6K1 region being derived from a strain of PSbMV able to overcome the resistance (Johansen *et al.*, 2001). Similar experimental strategies have been used to map determinants of *Turnip mosaic virus* (TuMV) symptom severity and avirulence on *Brassica napus* lines (Jenner *et al.*, 2002, 2003; Suehiro *et al.*, 2004) and the ability to infect radish (Suehiro *et al.*, 2004) to the P3 cistron. Collectively, these studies identify the P3 cistron as a key component of host–viral interactions specifying both pathogenicity and virulence.

WSMV is the type species of the genus *Tritimovirus* within the family *Potyviridae*, with a genome organization typical of a monopartite potyvirus (Stenger *et al.*, 1998). Five strains of WSMV have been completely sequenced (Stenger *et al.*, 1998; Choi *et al.*, 2001; Rabenstein *et al.*, 2002). Sequence comparisons among divergent WSMV strains revealed a substitutional ‘cold-spot’ within the 3′-proximal half of the P3 cistron (Choi *et al.*, 2001) that was atypically devoid of synonymous substitutions compared with the rest of the WSMV genome. A similar analysis (Choi *et al.*, 2001) revealed that five PVA strains (Kekarainen *et al.*, 1999) also retained high intraspecies sequence conservation within the 3′-proximal half of the P3 cistron. Because the 3′-proximal half of the P3 cistron displayed a high degree of conservation within but not between species, we hypothesized that this region of the genome may contain a species-specific RNA element that is retained among divergent species of the family. In this report, we describe mutational analyses of the WSMV-Sidney-81 genome, in which multiple synonymous substitution mutations were introduced into the putative P3 RNA element. Alterations of viral phenotype associated with synonymous substitutions in the P3 cistron were observed, providing evidence for the existence of an internal RNA element in the WSMV genome, and suggests that other potyviruses retaining high intraspecies sequence conservation in this region also may bear an RNA element with similar function.

## METHODS

**Construction of WSMV mutants.** A full-length infectious clone (pACYC-WSMV) of the Sidney-81 strain of WSMV from which infectious transcripts may be generated was described previously (Choi *et al.*, 1999). The full-length cDNA clone of WSMV-Sidney-81 also has been modified to include GUS coding sequences positioned at an engineered *SalI* site located immediately downstream of the WSMV P1 proteinase cleavage site (Choi *et al.*, 2002). Thus, pWSMV-GUS-S1RN (Fig. 1a) expresses functional GUS protein that is excised from the WSMV polyprotein upon amino-end proximal cleavage by the P1 proteinase and carboxy-end proximal cleavage by the NIa proteinase.

Site-directed mutagenesis within the P3 and CI cistrons was performed on a subclone of pACYC-WSMV containing the *SphI* fragment spanning nt 2373–4289 (Fig. 1a). Multiple synonymous substitution mutations were introduced into the 3′-proximal half of the P3 cistron to produce pP3M11, pP3M17, pP3M29, pP3M322 and pP3M4 (Fig. 1b). Multiple synonymous substitution mutations were introduced in the CI cistron to produce pCIM1 (Fig. 1b). Introduced substitutions were made taking codon usage patterns into account by



**Fig. 1.** WSMV genome organization and position of mutations introduced into the P3 and CI cistrons. (a) WSMV genome structure with shaded areas denoting regions targeted for mutagenesis. The location of the GUS reporter gene inserted into pWSMV-S1RN to produce pWSMV-GUS-S1RN is indicated. The positions and nucleotide coordinates of *SphI* restriction endonuclease sites to subclone a DNA fragment used as a mutagenesis template are indicated. The sequence of each mutant is listed in (b) relative to wild-type (WT) WSMV-Sidney-81 (pACYC-WSMV). Underlined bases denote nucleotides altered by mutagenesis. Codons are depicted as triplets with predicted amino acid translation listed by three letter code above each WT sequence. Unless indicated by black triangles, mutations are synonymous and do not affect translation of the encoded protein. Relevant nucleotide coordinates of WSMV-Sidney-81 are indicated in both (a) and (b). Sequences presented represent that of cDNA such that U is substituted for T in all RNA transcripts.

calculating relative synonymous codon usage (RSCU) values (Sharp *et al.*, 1986). An RSCU value of 1 for a codon indicates no bias in usage. Most of the synonymous substitutions introduced into the P3 cistron had RSCU values at or above 0.9. The lowest RSCU value for a synonymous substitution mutation in P3 resulted from change of a proline codon from CCG (RSCU = 0.81) to CCU (RSCU = 0.64). For CI, one serine codon substitution (AGC to UCC) altered RSCU from 1.11 to 0.35.

The *SphI* fragment of each mutagenized plasmid was completely sequenced to verify each mutant construct. Sequencing of mutagenized subclones revealed that two mutagenesis products contained additional unintended mutations. One plasmid (pP3M1) bore the same synonymous mutations as pP3M17 and one additional non-synonymous substitution, resulting in the alteration of an aspartic acid codon to an alanine codon (Fig. 1b). A second plasmid (pP3M3) bore the same synonymous mutations as pP3M322 but also incurred two separate single base deletions such that a frame-shift was introduced into the polyprotein open reading frame (ORF) (Fig. 1b). The *SphI* fragment of each mutagenized subclone was used to replace the wild-type *SphI* fragment of pACYC-WSMV to produce pWSMV-P3M11, pWSMV-P3M17, pWSMV-P3M29, pWSMV-P3M322, pWSMV-P3M4, pWSMV-P3M1, pWSMV-P3M3 and pWSMV-CIM1. The *SphI* fragment of mutagenized subclones was also used to replace the wild-type *SphI* fragment of pWSMV-GUS-S1RN, to place select mutations in a WSMV genome capable of expressing GUS. The resulting plasmids were designated pWSMV-G1-P3M17, pWSMV-G1-P3M29, pWSMV-G1-P3M322, pWSMV-G1-P3M4 and pWSMV-G1-P3M3.

To assess the effect of single synonymous substitutions in the P3 cistron, the four mutations present in pP3M11 (Fig. 1) were generated individually by site-directed mutagenesis of the *SphI* subclone bearing WSMV nt 2373–4289, verified by nucleotide sequencing, and used to replace the wild-type *SphI* fragment of pWSMV-GUS-S1RN. The resulting plasmids were designated pWSMV-G125 (A<sub>2755</sub> to T), pWSMV-G217 (A<sup>2761</sup> to G), pWSMV-G313 (C<sup>2770</sup> to T) and pWSMV-G41 (A<sup>2779</sup> to G).

**Coupled *in vitro* transcription/translation and immunoprecipitation.** *In vitro* transcription/translation products were synthesized from full-length WSMV cDNA sequences using the TnT SP6/wheat germ extract system (Promega) as per the manufacturer's recommendations, except that 6 µg plasmid template per 50 µl reaction mixture was used. A plasmid bearing the luciferase gene was used as a positive control for coupled *in vitro* transcription/translation reactions. *In vitro* translation products (45 µl) and 3 µl of diluted antiserum raised against the WSMV CI protein (Langenberg, 1993) or a synthetic peptide (LDTMASGAMKDYKIG) corresponding to the carboxy terminus of WSMV HC-Pro were added to 300 µl IP buffer (10% glycerol, 50 mM HEPES-KOH, 100 mM potassium glutamate, 0.5 mM DTT, 6 mM magnesium acetate, 1 mM EGTA, 0.1% NP40 and 0.5 mg ml<sup>-1</sup> BSA, pH 7.3) (Marcus *et al.*, 1994), and incubated (4 °C, 6 h) with gentle shaking. Protein A-Sepharose beads (5 mg) were added to 40 µl IP buffer; 40 µl of this slurry was added to the mixture of translation products and antiserum. Following incubation (4 °C for 4 h), the beads were washed five times with 400 µl IP buffer, resuspended in 20 µl gel loading buffer (50 mM Tris/HCl, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol, pH 6.8), and boiled for 3 min. Boiled protein samples were analysed on 12% SDS-PAGE followed by autoradiography.

**Infectivity assays.** *In vitro* SP6 transcripts were generated from *NotI* linearized plasmid DNA templates (12.5 µg) as described previously (Choi *et al.*, 1999) and mechanically inoculated onto leaves of 8–12-day-old wheat seedlings (cultivars Centurk or Arapahoe). Total RNA samples were extracted from non-inoculated, upper leaves of the primary shoot 21 days post-inoculation (p.i.). Systemic infection status of each plant was evaluated by RT-PCR using the primer CI-3 (5'-GTGGATCCTACTGGTATGACACACATGGG-3') for RT and the primers B-P1C4 (5'-GTCGGGATCCTGCGGATGATGCACTCCAAGGG-3') and P1-9-3 (5'-TTCCGAATTCCTAGTGCTTGCAAGAATGC-3') for PCR. The resulting RT-PCR product corresponded to WSMV-Sidney-81 nt 821–1461 and was detected by ethidium bromide staining after electrophoresis

in 1% agarose. Infectivity assays of WSMV transcripts bearing the GUS reporter gene were performed histochemically as described below.

**Protoplast preparation and transfection.** Protoplasts were prepared from barley leaves as described by Loesch-Freis & Hall (1980). Leaves from 8–10-day-old barley (cv. Larker) seedlings grown in a growth chamber (16 h photoperiod, 23 °C) were sliced into ~1 mm strips. Protoplasts were isolated from leaf strips by digestion (3 h, 30 °C) in enzyme mixture [10–20 mg Cellulysin cellulase (Calbiochem) ml<sup>-1</sup>, 1 mg Macerozyme R-10 (Yakult Pharmaceutical) ml<sup>-1</sup>, 1 mg BSA ml<sup>-1</sup>, 10% mannitol, pH 5.7]. Protoplasts were resuspended at ~5.5 × 10<sup>5</sup> ml<sup>-1</sup> in electroporation buffer (10% mannitol, 70 mM KCl, 5 mM MES, pH 5.7). *In vitro* transcription products (20 µg) were mixed with 400 µl resuspended protoplasts (~2.2 × 10<sup>5</sup> protoplasts) just prior to electroporation (200 V, 960 µF) in a 0.2 cm electrode cuvette using the Gene Pulser apparatus (Bio-Rad). Electroporated protoplasts were washed with 10% mannitol and incubated (36 h, 25 °C, 1000 lx continuous illumination) in 1 ml protoplast medium (Aoki & Takebe, 1969). Following incubation, protoplasts were stored frozen at -70 °C until processed for GUS activity using the fluorometric assay described below.

**GUS assays.** Histochemical detection of GUS expression was performed on small leaf pieces (1–1.5 cm length) of inoculated leaves 3 or 5 days p.i. (Choi *et al.*, 2000b). Leaf pieces were fixed for 1 h by vacuum infiltration in 0.7% formaldehyde. Fixed leaf pieces were rinsed five times (15 min each) in distilled water, then incubated (37 °C up to 12 h) in 50 mM phosphate buffer (pH 7.0) containing 2 mM 5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-gluc). Following incubation with X-gluc substrate, leaf pieces were clarified first in 70% ethanol and then in 5% sodium hypochlorite.

Fluorometric GUS assays were performed based on the methods of Jefferson *et al.* (1987). Protoplasts were thawed and pelleted by centrifugation (50 g, 2 min). Pelleted protoplasts were resuspended with 500 µl protein extraction buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.1% Triton X-100, 0.1% Sarcosyl, 1 mM EDTA, 5 mM DTT, pH 7.0). Glass beads (0.2 g, 450–650 µm diameter) were added to resuspended protoplasts and the mixture was vortexed for 1 min, followed by centrifugation (10 000 g, 10 min). Aliquots of the supernatant containing 20–50 µg total soluble protein were removed from each sample and adjusted to a volume of 300 µl with protein extraction buffer. Soluble protein samples were mixed with an equal volume (300 µl) of GUS assay buffer (2 mM 4-methylumbelliferyl β-D-glucuronide in protein extraction buffer) and incubated at 37 °C. Aliquots (100 µl) were removed at several time points between 0 and 120 min, measured for fluorescence (excitation at 365 nm, emission at 460 nm) using a TD-700 fluorometer (Turner Designs). Fluorescence values were converted to pmoles of methylumbelliferone (MU) min<sup>-1</sup> mg<sup>-1</sup> of soluble protein, utilizing known concentrations of MU as standards and a Bradford protein quantification (Bradford, 1976) assay kit (Bio-Rad). To account for variation between different protoplast preparations, each GUS activity measured was converted to a percentage value of that obtained with wild-type WSMV bearing GUS (pWSMV-GUS-S1RN) 36 h after transfection of protoplasts from the same preparation. Mean values of percentage GUS activity relative to wild-type were based on 4–6 replicates from independent protoplast preparations.

**RNA secondary structure.** Predictions of RNA secondary structure were generated using the program Alifold (Hofacker *et al.*, 2002), using an alignment of the five completely sequenced WSMV isolates. Folding free energies of predicted stem-loop structures were calculated using the methodology of Turner *et al.* (1988).

## RESULTS

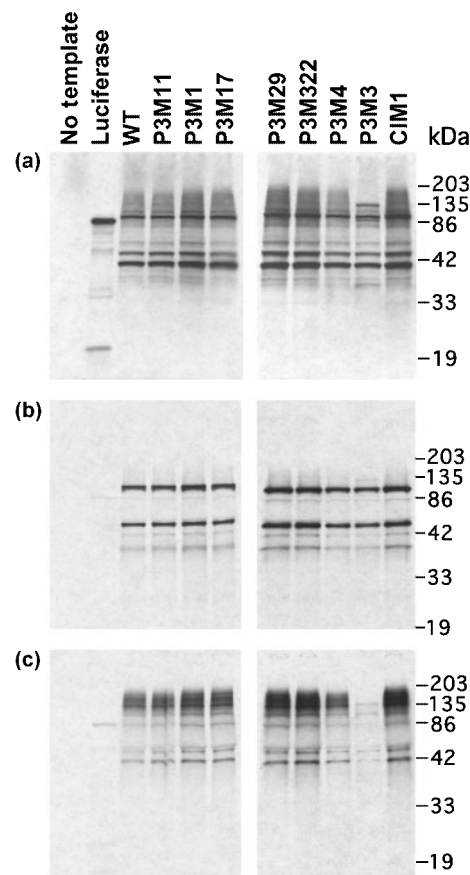
### *In vitro* translation of wild-type and mutant WSMV genomes

Because WSMV expresses all viral-encoded proteins via a single polyprotein with subsequent proteolytic cleavage, *in vitro* translation of genome-length RNA template yields complex products (Fig. 2). Nonetheless, the pattern and amount of *in vitro* translation products of wild-type WSMV and all mutants examined were essentially identical, with the exception of the frame-shift mutant pWSMV-P3M3 (Fig. 1). The most noticeable difference in translation of the frame-shift mutant was the absence of high molecular mass ( $\geq 135$  kDa) polypeptide products (Fig. 2a).

Immunoprecipitation of *in vitro* translation products with antiserum to HC-Pro (Fig. 2b) resulted in similar profiles of recovered proteins for wild-type and all mutants. The two major protein products immunoprecipitated by HC-Pro antibodies were interpreted as fully cleaved HC-Pro protein and a larger P1–HC-Pro fusion. Because the frame-shift in pWSMV-P3M3 is located downstream of the HC-Pro/P3 junction, precipitation of identical *in vitro* translation products was as expected. In contrast, immunoprecipitation of *in vitro* translation products with CI antibodies did result in discrimination of the frame-shift mutant from all other WSMV genomes translated (Fig. 2c). Minor amounts of product immunoprecipitated with CI antibody from the *in vitro* translation reaction programmed with the frame-shift mutant pWSMV-P3M3 most likely represents an internal initiation at in-frame AUG codons positioned in the WSMV genome downstream of the frame-shift mutation. Importantly, yields of translation products precipitated by CI antibodies were essentially the same for wild-type and all other mutants (Fig. 2c), indicating that the introduced upstream synonymous substitutions had little or no effect on translation.

### Systemic infectivity of mutants in wheat

Wild-type WSMV transcripts derived from pACYC-WSMV were infectious in wheat (Table 1) and produced characteristic systemic mosaic symptoms. Transcripts derived from pWSMV-CIM1 bearing 15 synonymous nucleotide substitutions in the CI cistron (Fig. 1) were also infectious and induced systemic symptoms (Table 1). In contrast, plants inoculated with transcripts of all P3 multiple synonymous substitution mutants (Fig. 1) produced no symptoms during 30 days p.i. (Table 1). To detect potential asymptomatic systemic infection, all inoculated plants were evaluated for the presence of WSMV-specific sequences in non-inoculated leaves by RT-PCR at 21 days p.i. All asymptomatic plants tested negative for WSMV infection, whereas all symptomatic plants tested positive for WSMV infection (data not shown). To ensure that lack of systemic infectivity was due to the introduced P3 mutations, the wild-type *SphI* fragment (originally subcloned for use as a mutagenesis template) was used to replace the mutant *SphI*



**Fig. 2.** *In vitro* translation of wild-type (WT) and mutant WSMV transcripts. (a) Autoradiograph of <sup>35</sup>S-labelled translation products in a wheat germ system programmed with WT RNA transcribed from pACYC-WSMV or mutant derivatives of pACYC-WSMV bearing multiple synonymous mutations in the P3 cistron (P3M11, P3M17, P3M29, P3M22 and P3M4), multiple synonymous mutations in the CI cistron (CIM1), silent mutations and 1 aa substitution in the P3 cistron (P3M1), or frame-shift mutations in the P3 cistron (P3M3). *In vitro* translation performed in the absence of exogenous template (no template) served as a negative control. *In vitro* translation performed with RNA transcribed from the luciferase gene (luciferase) served as a positive control. (b) Autoradiograph of translation products from (a) after immunoprecipitation with antibodies raised against a synthetic peptide corresponding to the amino terminus of WSMV HC-Pro protein. (c) Autoradiograph of translation products from (a) after immunoprecipitation with antibodies raised against WSMV CI protein. All samples were subjected to SDS-PAGE prior to autoradiography. The mobility and sizes (kDa) of protein standards are indicated on the right.

fragment of pWSMV-P3M17 (Fig. 1). This replacement restored infectivity and systemic symptom production, demonstrating that no unintended debilitating mutations had occurred in WSMV sequences outside of the *SphI* fragment.

**Table 1.** Systemic infection of wheat with *in vitro* transcripts from WSMV full-length clone and mutant derivatives

Inoculum template	Experiment 1* cv. Centurk	Experiment 2* cv. Arapahoe
pACYC-WSMV†	7/10	14/14
pWSMV-P3M1‡	0/12	0/11
pWSMV-P3M11§	0/15	0/19
pWSMV-P3M17§	0/12	0/16
pWSMV-P3M29§	0/13	0/18
pWSMV-P3M3	0/16	0/18
pWSMV-P3M322§	0/12	0/14
pWSMV-P3M4§	0/13	0/13
pWSMV-CIM1§	8/14	12/17

\*No. of plants systemically infected/no. of plants inoculated.

†Wild-type.

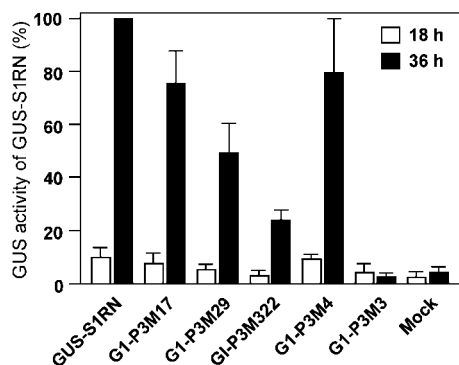
‡Synonymous mutations plus one non-synonymous mutation.

§Synonymous mutations only.

||Synonymous mutations plus frame-shift mutations.

### Replication of wild-type and mutant WSMV genomes in protoplasts

The GUS gene inserted between P1 and HC-Pro was used as a reporter to indirectly compare replication of wild-type and mutant WSMV transcripts in barley protoplasts (Fig. 3). GUS activity measured 36 h post-transfection indicated that the P3 mutants bearing multiple synonymous mutations maintained GUS activity at 22–80 % of wild-type WSMV bearing GUS. Transcripts derived from G1-P3M322 showed the lowest GUS activity among mutants tested, indicating



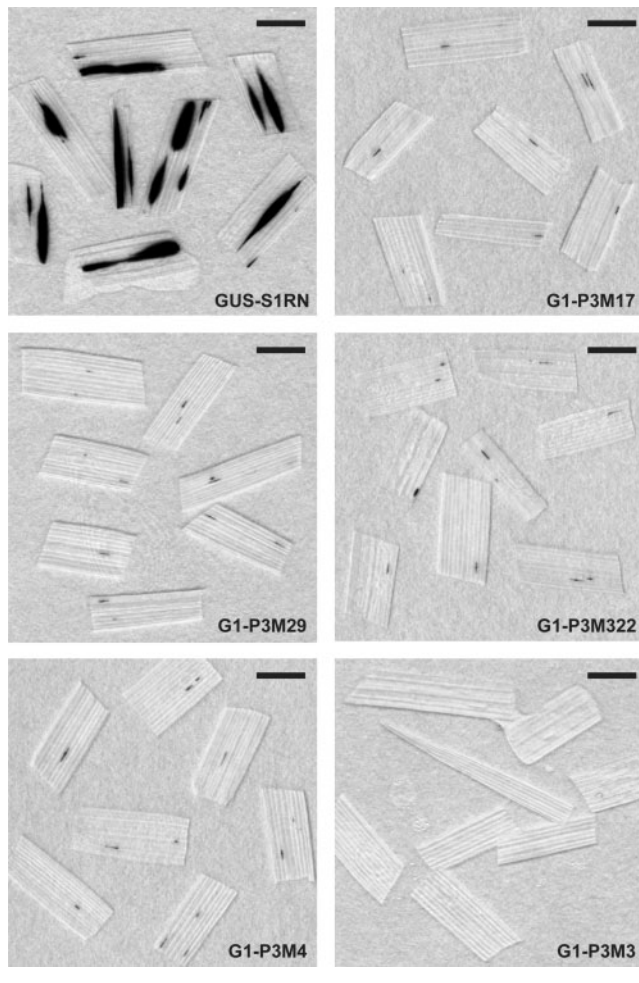
**Fig. 3.** Replication of wild-type and mutant WSMV bearing the GUS reporter gene in barley protoplasts 36 h post-transfection. GUS expression was measured by fluorescence and calculated as pmoles MU min<sup>-1</sup> mg<sup>-1</sup> protein. To account for variation between experiments with different protoplast preparations, GUS expression of each sample was normalized as a percentage (%) of wild-type WSMV (GUS-S1RN) expressing GUS at 36 h post-transfection. Bars represent mean percentage GUS expression relative to wild-type and were based on 4–6 replicates per treatment. Error bars represent standard deviation.

the replication of the mutant was severely affected by the synonymous substitutions near nt 2950–2980. Similarly, the P3 mutant also bearing two single base deletions (pWSMV-G1-P3M3), resulting in a frame-shift of the polyprotein ORF, expressed GUS activity at only 2 % of wild-type levels. Because WSMV utilizes a polyprotein expression strategy, the frame-shift not only affected the P3 protein, but also resulted in polar mutation of all downstream cistrons. Thus, the carboxy-terminal portion of P3 was translated in a different frame ending at a stop codon (UGA), corresponding to WSMV-Sidney-81 nt 3163–3165. Given that this frame-shift would prevent translation of the cistrons encoding 6K1, CI, 6K2, NIa, NIb and CP, it was expected that such a transcript would not produce a replication-competent genome. Therefore, we interpreted intermediate levels of GUS activity, observed for mutant genomes bearing only synonymous mutations in P3, as evidence of genome amplification in this transient assay.

### Establishment of infection foci in inoculated wheat leaves

Because the P3 synonymous mutants were unable to systemically infect wheat plants (Table 1) but did not display altered *in vitro* translation properties (Fig. 2) and could replicate (albeit with reduced efficiency) in barley protoplasts (Fig. 3), we speculated that the synonymous substitution mutations in P3 may affect viral movement. To assess movement phenotype, histochemical GUS assays of inoculated leaves were performed 3 days p.i. with transcripts from cDNA templates bearing GUS in wild-type or mutant WSMV genomes (Fig. 4). Large but discrete patches of GUS expression were observed 3 days p.i. on leaves inoculated with wild-type WSMV bearing GUS (pWSMV-GUS-S1RN). In contrast, infection foci identified by GUS expression 3 days p.i. on leaves inoculated with each P3 multiple synonymous substitution mutant consisted of small clusters of cells (Fig. 4). No GUS activity was observed 3 days p.i. in leaves inoculated with the frame-shift mutant (pWSMV-G1-P3M3). Histochemical assay of GUS expression in inoculated leaves 5 days p.i. indicated that infection foci of wild-type WSMV expressing GUS had continued to expand and coalesce, whereas no GUS activity was detected 5 days p.i. in leaves inoculated with the frame-shift mutant (data not shown). In contrast, infection foci for all P3 multiple synonymous substitution mutants remained discrete and only slightly increased in size by 5 days p.i. (data not shown).

The synonymous mutations present in pP3M11 were reconstructed as single mutations in pWSMV-GUS-S1RN and evaluated for infectivity and movement phenotype (Fig. 5). Three of the four single mutations resulted in wild-type systemic infection. Only one single mutation (G217 with A<sup>2761</sup> to G) did not produce a systemic infection and was identical to P3M11 with respect to movement phenotype, in which infection foci were limited to small clusters of cells within inoculated leaves (Fig. 5). Nucleotide sequencing of RT-PCR clones derived from plants

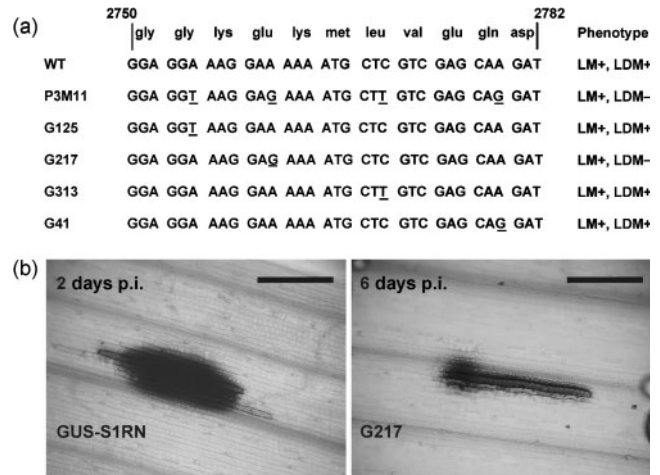


**Fig. 4.** Movement of wild-type WSMV and mutants with multiple synonymous substitutions in the P3 cistron. Expression of GUS due to virus infection in wheat leaves were examined 3 days p.i. with wild-type or mutant WSMV transcripts bearing the GUS reporter gene. Presented are images of wheat leaf pieces in which dark stain denotes regions of GUS expression that define individual infection foci. Bars, 5 mm. Note that infection foci of wild-type WSMV (WSMV-GUS-S1RN) were much larger than those produced by genomes bearing multiple synonymous substitution mutations in P3 (G1-P3M17, G1-P3M29, G1-P3M322 and G1-P3M4), and that no GUS expression was detected in leaves inoculated with a frame-shift mutant (G1-P3M3).

systemically infected with the single mutants G125, G313 and G41 confirmed that each of these mutants was stable and that systemic infectivity was not due to reversion (data not shown).

### Predicted RNA secondary structures for wild-type and P3 mutants

Arbitrarily introduced synonymous substitutions spanning over 330 nt of the P3 cistron resulted in similar debilitating phenotypes. To determine if this may be due to structural

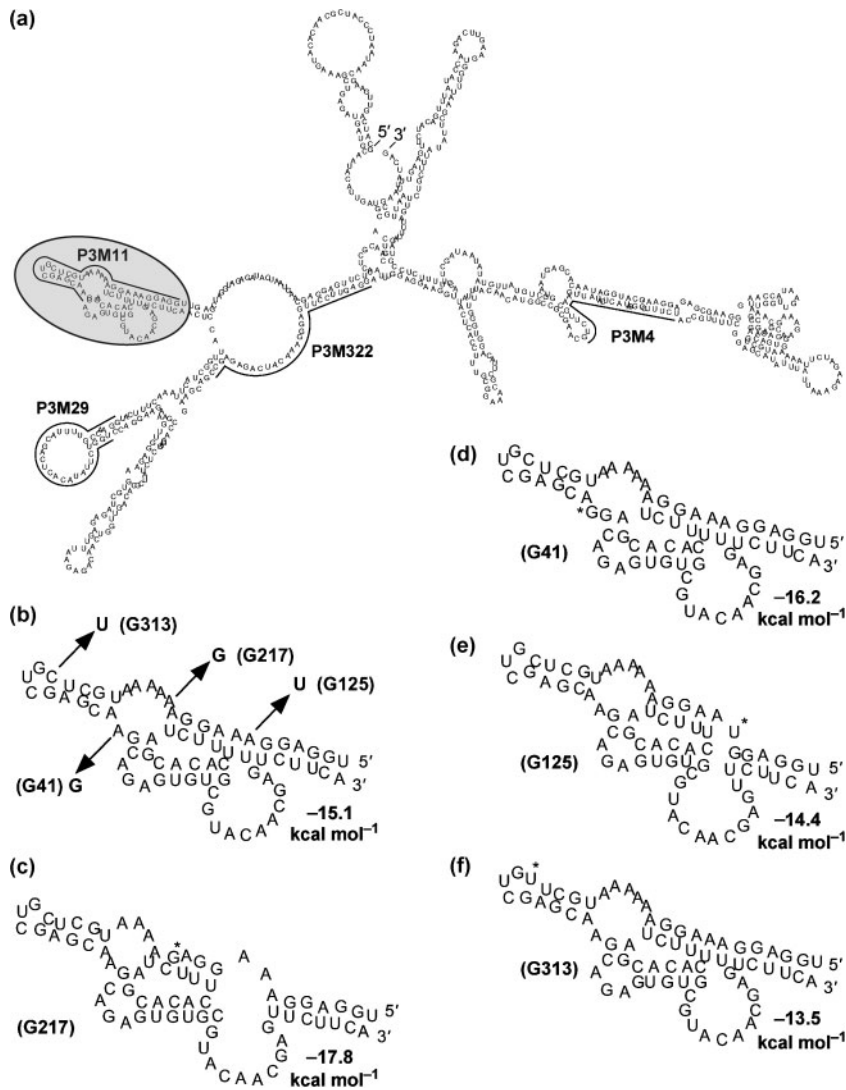


**Fig. 5.** Movement phenotypes of WSMV mutants bearing an individual synonymous substitution in the P3 cistron. The sequence (as DNA template) of the multiple synonymous substitution mutant P3M11 and each individual synonymous substitution mutant (G125, G217, G313 and G41) are listed in (a) relative to wild-type (WT) WSMV-Sidney-81. Underlined bases denote nucleotides altered by mutagenesis. Codons are depicted as triplets with predicted amino acid translation listed by three letter code above the WT sequence. Relevant nucleotide coordinates of WSMV-Sidney-81 are indicated above (a). The movement phenotype (LM, local cell-to-cell movement; LDM, long-distance systemic movement) of each synonymous substitution mutant is given at the right of (a). (b) Light micrographs of histochemical staining for GUS activity at infection foci produced on inoculated wheat leaves by wild-type WSMV-Sidney-81 bearing GUS (GUS-S1RN) or the single synonymous substitution mutant G217 that was capable of LM but not LDM. Bar, 0.5 mm.

constraints on the RNA sequence, we sought to determine whether the five WSMV genomic sequences, which originally brought to light the relative paucity of sequence diversity in P3, had a common predicted structure. Use of the Alifold computer program (Hofacker *et al.*, 2002) revealed a secondary structure involving the entire 'cold spot' sequence (Fig. 6). All four mutated regions shown in Fig. 1 would disrupt elements of this predicted structure. Strikingly, none of the single substitutions in the infectious mutants G125, G313 and G41 (Fig. 5) significantly altered secondary structure (Fig. 6b, d–f), whereas the A<sup>2761</sup> to G substitution of mutant G217 changed both a stem and two bulge loops, resulting in a more stable structure ( $\Delta G^\circ = -17.8 \text{ kcal mol}^{-1}$  versus  $\Delta G^\circ = -15.1 \text{ kcal mol}^{-1}$  for wild-type; Fig. 6c).

## DISCUSSION

Based on sequence comparisons among divergent strains of WSMV, we hypothesized that the paucity of synonymous substitutions in the 3'-proximal half of the P3 cistron



**Fig. 6.** Secondary RNA structure of the WSMV P3-6K1 region. (a) Depicts secondary structure for the entire substitutional cold-spot region (nt 2633–3325) predicted to be conserved among five WSMV strains. Sequences defined by black lines (P3M11, P3M29, P3M322 and P3M4) were subjected to multiple synonymous substitution mutagenesis, which significantly altered predicted secondary structure (not shown) and abolished systemic infectivity. (b) Shows an enlarged view of wild-type secondary structure and specific synonymous mutations introduced into P3M11 [highlighted by grey oval in (a)]. (c–f) Display altered secondary structures for individual substitution mutants (G217, G41, G125 and G313, respectively) with asterisks denoting the substitution in each structure. Calculated folding free energies ( $\Delta G^\circ$ ) are provided below each of the structures shown in (b–f). Note that of the four single substitution mutations in P3M11, only the single substitution mutant G217 ( $A^{2761}$  to G) significantly altered secondary structure and abolished systemic infectivity.

was due to the presence of an essential and previously unrecognized RNA sequence element. The experiments presented in this report support our hypothesis, as WSMV genomes bearing only synonymous substitutions in P3 were impaired with respect to movement and replication, whereas multiple synonymous substitutions in CI had no phenotypic effect. The introduced substitutions were made taking care not to significantly alter codon usage, and indeed no differences were observed among translation products derived from wild-type or mutant genomes bearing synonymous substitutions in the P3 or CI cistrons. Of particular interest was the P3 single synonymous substitution mutant G217, which differed from wild-type only in the alteration of a glutamic acid codon from GAA to GAG. For WSMV-Sidney-81, the polyprotein ORF utilizes the codon GAA in 101 positions (RSCU=1.07) and the alternative codon GAG in 88 positions (RSCU=0.93). In P3 itself, there are 11 GAG codons and 12 GAA codons. These observations clearly demonstrate that use

of either codon specifying glutamic acid is tolerated by WSMV, such that the long-distance movement-defective phenotype of P3 mutant G217 cannot be explained as resulting from codon usage bias.

Information encoded by viral RNA genomes is not limited to codons specifying the order of amino acids incorporated into viral proteins. RNA elements direct a variety of essential functions critical to the viral life cycle and, although most act in *cis*, several viral RNA elements function in *trans* (Sit *et al.*, 1998; Shen & Miller, 2004). Although *cis*-acting RNA elements (CREs) involved in viral RNA polymerase template recognition and regulation of replication are typically associated with the non-coding regions (NTR) situated at the ends of viral RNA genomes, these elements may extend into adjacent coding regions. Other CREs may be found entirely (or nearly so) within coding regions. Species-specific CREs located in coding regions have been identified in several viruses of the *Picornaviridae* (Mcknight

& Lemon, 1996; Lobert *et al.*, 1999; Goodfellow *et al.*, 2000; Gerber *et al.*, 2001). It was shown that these CREs function as template for uridylylation of the genome-linked protein (VPg) (Rieder *et al.*, 2000; Yang *et al.*, 2002), and that the product of uridylylation, VPgpUpU, serves as a primer for the synthesis of positive-strand RNA (Goodfellow *et al.*, 2003; Morasco *et al.*, 2003; Murray & Barton, 2003). The current model for picornavirus replication suggests that replication is coordinated by interactions among multiple *cis*-elements in 5'- and 3'-NTRs and coding regions (Barton *et al.*, 2001; Lyons *et al.*, 2001; Goodfellow *et al.*, 2003; Morasco *et al.*, 2003; Murray & Barton, 2003). Based on the similarity in genome structures, WSMV and viruses of the *Potyviridae* belong to the picornavirus superfamily. CREs necessary for efficient amplification of the genome have been identified in the capsid protein (CP) coding region and 3'-NTR of *Tobacco etch virus* (TEV) (Mahajan *et al.*, 1996; Haldeman-Cahill *et al.*, 1998). Collectively, our results indicate that another CRE resides in the P3 cistron.

Systemic movement of plant viruses occurs by a two-step process: cell-to-cell movement between adjacent cells through plasmodesmata followed by long-distance movement via phloem. Mutational analyses of potyvirus genomes and ultrastructural studies of infected tissues indicated that several viral-encoded proteins are involved in movement (Dolja *et al.*, 1994, 1995; Klein *et al.*, 1994; Kasschau *et al.*, 1997; Rojas *et al.*, 1997; Carrington *et al.*, 1998; Lopez-Moya & Pirone, 1998). In the case of WSMV, the P3 synonymous substitution mutants were able to initially spread from cell-to-cell. However, after several days p.i. cell-to-cell movement of the P3 mutants appeared to cease with infection foci limited to small clusters of cells. Thus, long-distance movement may be precluded indirectly by limitation of cell-to-cell movement such that infection foci fail to reach phloem-associated parenchyma. This phenotype is distinct from subliminal infections limited to single cells that result from a point mutation in the TEV CP (Dolja *et al.*, 1995) or from truncation of the WSMV CP (unpublished data). In many respects, the debilitated movement phenotype of WSMV P3 synonymous substitution mutants resembles that of long-distance movement deficient TEV HC-Pro mutants (Kasschau *et al.*, 1997) or a TEV CP mutant lacking 17 aa of the carboxy terminus (Dolja *et al.*, 1995).

Interestingly, the A<sup>2761</sup> to G substitution of mutant G217 altered the predicted secondary structure (Fig. 6c), while the single substitutions in the infectious mutants G125, G313 and G41 did not (Fig. 6b, d–f). At present we do not have informative data with respect to how perturbation of P3 RNA secondary structure affects WSMV replication and movement. We attempted to complement a movement-deficient P3 mutant bearing GUS by co-inoculation with a wild-type WSMV genome. No GUS activity was observed in systemically infected tissues (data not shown), suggesting that the element does not act in *trans*. We further examined whether the position of the P3 RNA element was important. In this experiment, we placed the wild-type P3 cistron

between the NIb and CP cistrons in a genome also bearing multiple synonymous mutations in the resident P3 cistron. Infectivity was not restored (data not shown), indicating that the position of the RNA element was critical. This suggests a fundamental difference between the P3 element and poliovirus CRE, because the latter retains function independent of position (Goodfellow *et al.*, 2000). Because there is limited experimental evidence that encapsidation of potyviral genomes may initiate in the 5'-proximal half of genome (Wu & Shaw, 1998), we speculated that the P3 RNA element may be required for virion assembly. To address this issue, we examined extracts from transfected protoplasts for virions by electron microscopy (data not shown). Unfortunately, the protoplast system employed here was suboptimal, as we were unable to observe virions in extracts derived from protoplasts transfected with any WSMV construct (including wild-type), precluding meaningful analysis. Clearly, additional experimentation is necessary to define the mechanism(s) by which the WSMV P3 RNA element affects replication and movement.

Although the biochemical function of P3 protein is still unclear, mutant analysis (Klein *et al.*, 1994) and interactions between P3 and other proteins encoded by potyvirus genomes (Choi *et al.*, 2000a; Merits *et al.*, 1999; Rodríguez-Cerezo *et al.*, 1993) suggest an involvement of P3 in the replication process. Previous studies elegantly demonstrated that the potyvirus P3 cistron is an important determinant of pathogenicity and virulence (Sáenz *et al.*, 2000; Johansen *et al.*, 2001; Hjulsgager *et al.*, 2002; Jenner *et al.*, 2002, 2003; Suehiro *et al.*, 2004). In each case, the differential phenotype mapped to the P3 region was presumed to be due to differences in the P3 protein based on comparative sequence analysis or site-directed mutagenesis, in which non-synonymous mutations altered phenotype. However, based on our data, it is apparent that the primary RNA sequence of the WSMV P3 cistron plays an essential role in the viral life cycle and that it is necessary to distinguish among functions of the P3 coding region governed by RNA elements from those of the encoded protein. The secondary structure model for the region presented in Fig. 6 can be tested by additional physical and mutagenesis studies. At this time it is unknown if the genomes of divergent species of the family *Potyviridae* also harbour an essential CRE within the P3 coding sequence. Similar mutagenesis studies with infectious potyvirus clones would reveal whether the P3 CRE is retained among monopartite members of the family or is peculiar to the tritrovirus WSMV.

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